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Alloimmunity and tolerance in corneal transplantation

Afsaneh Amouzegar*, Sunil K. Chauhan*,², and Reza Dana*,²

*Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts 02114

Abstract

Corneal transplantation is one of the most prevalent and successful forms of solid tissue transplantation. Despite favorable outcomes, immune-mediated graft rejection still remains the major cause of corneal allograft failure. While ‘low risk’ graft recipients with uninflamed graft beds enjoy a success rate of approximately 90%, the rejection rates in inflamed graft beds or ‘high risk’ recipients often exceed 50% despite maximal immune suppression. In this review we discuss the critical facets of corneal alloimmunity, including immune and angiogenic privilege, mechanisms of allosensitization, cellular and molecular mediators of graft rejection, and allotolerance induction.

Keywords

Cornea; Transplantation; Alloimmunity; Tolerance; Regulatory T cells

Introduction

More than a century has passed since Eduard Zirm, an Austrian ophthalmologist, performed the first partially successful full-thickness human corneal transplant (1). Today, corneal transplantation is one of the most prevalent forms of solid tissue transplantation performed in the world (2). It is estimated that well over 100 000 corneal transplant surgeries are performed annually worldwide, with nearly 40 000 performed annually in the United States alone (3). Several trends are notable. First, the number of corneal grafts performed in the developing world, especially Asia, is rising sharply due to enhanced eye banking procedures and distribution networks. Second, partial-thickness corneal transplants (lamellar keratoplasty) are increasingly being used in place of full-thickness transplants (penetrating keratoplasty) when the entire cornea does not need to be replaced, and this trend has led to some decreased risk of graft rejection, likely due to decreased load of allogeneic tissue (4, 5). Still, it is critical to emphasize that the most important prognosticator of graft success is the status of the recipient bed in which the corneal graft is placed. The 2-year graft survival for penetrating keratoplasty in non-vascularized and uninflamed host beds or ‘low risk’

CORRESPONDENCE: Sunil K. Chauhan, DVM, PhD (; Email: sunil_chauhan@meei.harvard.edu), Phone: 617-912-0258; Fax: 617-912-0117, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114 or Reza Dana, MD, MSc, MPH (; Email: reza_dana@meei.harvard.edu), Phone: (617) 912-7401, Fax: (617)-912-0117, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114.

²S.K.C. and R.D. are equally contributing corresponding authors.

corneal transplants is approximately 90% (6). However, recipients with a previous history of graft rejection or grafts performed in inflamed and vascularized host beds are considered to be at high risk of rejection, with failure rates of over 50% despite maximal local and systemic immunosuppressive therapy (7) – outcomes that are considerably worse than kidney, heart or liver transplants. Interestingly, the remarkable rate of success normally seen in low-risk corneal grafts, unlike other solid transplants, is achievable without the benefit of HLA matching or profound systemic immune suppression (7).

Despite these favorable outcomes, graft rejection remains the leading cause of corneal allograft failure (8). Corneal graft rejection can occur in any of the three cell layers of the cornea—epithelium, stroma or endothelium, with endothelial rejection being the most prevalent sight-threatening form, which can be attributed to the fact that the endothelial cells of the cornea are irreplaceable, and perform the critical function of preventing the tissue from getting swollen (9). Graft rejection is clinically characterized by graft edema (swelling) and inflammatory cells that can be seen circulating in the anterior chamber of the eye or attaching as keratic precipitates to the graft endothelial cells (Fig 1A) (10). Several factors have been associated with a higher risk of graft failure, with the degree of host bed neovascularization being the most significant prognosticator for earlier and a more severe graft rejection (7, 11, 12).

In this review, we discuss the factors involved in ocular inflammation, activation and migration of antigen-presenting cells (APCs), pathways of allosensitization and allotolerance, and mechanisms of graft destruction (Fig 1B).

Immune and angiogenic privilege of the cornea and their implications in transplant immunity

The term “immune privilege” was first coined by Sir Peter Medawar in the late 1940s, when he recognized the extended survival of skin allografts placed in the anterior chamber of the eye (13). He attributed this unexpected graft survival to what he considered to be “immunological ignorance”, a passive process of sequestration of foreign antigens in the anterior chamber due to the absence of draining lymphatic vessels. However, later in the 1970s, Kaplan and Streilein demonstrated that immune privilege was in fact the result of an actively maintained and “deviant” suppressive immune response to ocular antigens (14), a phenomenon that was later referred to as anterior chamber-associated immune deviation (ACAID) (15).

Immune Privilege—ACAID is a form of immune tolerance to alloantigens placed in the anterior chamber of the eye that results in downregulation of antigen-specific delayed type hypersensitivity (DTH) response, while promoting humoral immunity and production of non-complement fixing antibodies (16). This process is thought to be mediated by F4/80⁺ APCs in the eye that capture intraocular antigens, enter the bloodstream and migrate to the marginal zone of the spleen, where their interactions with CD4⁺ T cells, $\gamma\delta$ T cells, B cells, and natural killer (NK) T cells result in the generation of two groups of antigen-specific regulatory T cells (Tregs): CD4⁺ CD25⁺ Tregs and CD8⁺ Tregs (17). Studies on the implication of ACAID in murine models of corneal transplantation have demonstrated that

ACAID induction through intraocular injection of allogeneic lymphocytes prior to penetrating keratoplasty causes a significant improvement in corneal allograft survival (18, 19).

Aside from immunological tolerance induced by ACAID, several other mechanisms contribute to the maintenance of ocular immune privilege (Table 1). The cornea expresses an array of membrane-bound immunomodulatory molecules that protect the cornea from inflammation and promote immune quiescence. Programmed death ligand-1 (PDL-1) is a pro-apoptotic molecule, which is constitutively expressed at high levels by the cornea (20, 21). PDL-1 interaction with PD-1 receptor on T cells results in inhibition of T cell proliferation, apoptosis induction and inhibition of IFN γ production leading to prolonged allograft survival (20, 22). Recent studies have established distinct mechanisms for donor and host derived PDL-1 in promoting corneal allograft survival. Depletion of PDL-1 in graft recipients results in considerably stronger indirect T cell priming and rapid graft rejection than wild-type recipients (20). Elimination of PDL-1 in the graft donor, however, does not have a significant effect on indirect allorecognition, but results in an increased alloreactive T cell infiltration and graft failure (20). IL-1Ra, which is constitutively expressed by the cornea, promotes immune privilege through suppression of antigen-presenting cell migration to the cornea (23). Topical administration of IL-1Ra has been shown to promote allograft survival in both low risk and high risk graft recipients in a murine model of corneal transplantation (24). Thrombospondin-1 (TSP-1) is an immunomodulatory glycoprotein expressed by the cornea and immune cells such as APCs. Studies have demonstrated significantly higher rates of graft rejection in TSP-1 null mouse corneal allografts compared to wild-type grafts (25). APC-derived TSP-1 inhibits maturation of APCs during inflammation, regulates their migration to draining lymph nodes, and suppresses their capacity in direct sensitization of alloreactive T cells (25). Corneal epithelial and endothelial cells constitutively express the pro-apoptotic molecule FasLigand (CD95L). Interaction of FasLigand with Fas (CD95)-expressing inflammatory cells such as neutrophils and effector T cells results in their apoptotic death, thereby improving allograft survival (26). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) is a transmembrane protein highly homologous with FasL which is constitutively expressed by corneal cells (27). TRAIL has been implicated in apoptosis of activated T cells and has been found to promote the proliferation of Tregs (28). Although no studies have directly associated TRAIL expression with corneal graft survival, transfection of mouse donor corneas with adenovirus carrying TRAIL gene has been shown to significantly improve corneal allograft survival (29). In addition to these membrane-bound proteins, several soluble immunoregulatory factors are found in the aqueous humor of the eye including TGF- β_2 , complement regulatory proteins (CRP), alpha-melanocyte stimulating hormone (α -MSH), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), somatostatin, macrophage migration inhibitory factor (MIF) and indoleamine 2, 3-dioxygenase (IDO), which are involved in tolerance induction in macrophages, regulation of dendritic cells, and inhibition of complement and NK cell-mediated cell lysis and T cell activation (30–39).

Angiogenic Privilege—The cornea is devoid of vasculature and lymphatics, which limits the trafficking of immune cells between the cornea and the systemic circulation and

lymphoid organs. This “angiogenic privilege” is actively maintained through expression of several anti-angiogenic factors. Corneal epithelial cells constitutively secrete soluble VEGFR-1 which binds VEGF-A, and thus inhibits its mitogenic effect on vascular endothelial cells (40). Additionally, non-signaling VEGFR-3, which is constitutively expressed by the corneal epithelium, demonstrates antiangiogenic activity by acting as a decoy receptor for VEGF-C and VEGF-D (41, 42), thereby suppressing both blood and lymphatic vessel growth. Studies on a mouse model of corneal transplantation have also demonstrated that blockade of VEGFR-3 or VEGFR-3 binding ligands such as anti-VEGF-C results in significant inhibition of lymphangiogenesis and ultimately prolonged corneal graft survival (43). Corneal epithelial cells additionally express soluble VEGFR-2 that inhibits VEGF-C and thus blocks lymphangiogenesis (44). Intracorneal administration of soluble VEGFR-2 has been shown to double allograft survival in a mouse model of corneal transplantation (44). Other anti-angiogenic factors that are expressed by the cornea include (i) angiostatin, which inhibits vascular endothelial cell proliferation and migration (45); (ii) endostatin, which blocks the mitogenic activity of VEGF on vascular endothelial cells and promotes their apoptosis (46); (iii) pigment epithelium-derived factor (PEDF) expressed by corneal epithelium and endothelium that exerts its anti-angiogenic effect through downregulation of VEGF expression (47); and (iv) thrombospondin-1 (TSP-1), which inhibits hemangiogenesis and lymphangiogenesis by induction of vascular endothelial cell apoptosis and CD36-mediated downregulation of VEGF-C (48). Results of a recent study have shown that subconjunctival administration of endostatin to mice undergoing corneal transplantation inhibits graft neovascularization and T cell infiltration, and significantly prolongs corneal graft survival (49). It has also recently been demonstrated that PD-L1 has anti-angiogenic functions (50). Suture-induced corneal inflammation in PD-L1 knock out mice results in a more significant angiogenic response and higher levels of VEGFR-2 expression compared to wild-type mice (50). This complex interplay between anti- and pro-angiogenic factors demonstrates the importance of regulation of hem- and lymphangiogenesis in the maintenance of corneal immune privilege.

Relevance of major histocompatibility complex (MHC) antigens in corneal alloimmunity

All corneal cells including epithelial, stromal and endothelial cells express MHC class I antigens. Interestingly, in the healthy cornea only DCs in the peripheral cornea express MHC class II, while DCs and other myeloid cell populations residing in the center of the cornea express minimal to no levels of MHC class II (51). In contrast to their kin in the skin, inflammatory stresses induce MHC class II expression by the significant majority of corneal DCs and corneal epithelial cells (30).

HLA (MHC) matching along with systemic immunosuppression are the cornerstones of prophylactic strategies against rejection for solid organ transplants such as the kidney; however, neither is routinely performed in corneal transplantation. A myriad of studies have been performed since the 1970s on relative efficiency of HLA matching in corneal allograft survival. The results from these studies have been mixed, with some studies showing a clear benefit (52, 53). However, the largest prospective randomized study of HLA matching in corneal transplantation in the United States, the Collaborative Corneal Transplantation Studies (CCTS), showed no benefit for HLA-DR matching (7). Evidence regarding the

effect of MHC class II/HLA-DR matching remains unclear. Many studies have demonstrated prolonged survival rates in grafts with lower number of HLA-DR mismatches, especially in the high-risk setting (52, 54). This is in contrast with the CCTS, which reported no significant difference in graft rejection rates between cross-matched and non HLA-matched groups (7). The failure of CCTS to demonstrate beneficial results for HLA-matching on graft survival has been attributed to various factors, including the possibility of incorrect typing (these studies were primarily done in the 1980s before the advent of DNA-based typing methods) and aggressive immunosuppressive therapy regimens that can nullify the beneficial effects of HLA matching, in part by suppressing MHC expression (55). The CCTS, however, reported that matching for minor histocompatibility antigens such as ABO group may improve graft survival. In humans, donor-recipient matching of minor H-Y antigen has recently been associated with significantly lower graft rejection rates (56). The role of minor histocompatibility antigens is more prominent in low risk corneal transplants, where resident corneal APCs express minimal levels of MHC class II, as described above. However, significantly increased expression of MHC class II and co-stimulatory molecules (e.g. CD80, CD86, CD40) by graft APCs in inflamed host beds leads to activation of the direct pathway of allosensitization, further underscoring the possible utility of MHC matching in the high-risk setting (57, 58). Despite the preponderancy of data suggesting that MHC class II matching can enhance corneal allograft survival in high risk grafts, it is likely that given its high economic costs, the debate regarding the merits of MHC matching in corneal transplantation will continue until large randomized clinical trials using modern DNA-based typing methods provide more definitive answers.

Mechanisms of allosensitization: significance of direct versus indirect allorecognition pathways

Corneal graft rejection is a complex process during which changes in the corneal microenvironment and the interplay between cells of the innate and adaptive immunity result in graft destruction. Early after transplantation, ocular surface inflammation leads to upregulation of proinflammatory cytokines such as IL-1, IL-6, and TNF- α (59), chemokines including MIP-1 α , MIP-1 β , MIP-2, and RANTES (60), and overexpression of adhesion molecules such as ICAM-1 and VLA-1 (61–63). This inflammatory milieu results in the acquisition of high levels of MHC class II and costimulatory molecules such as CD80 (B7-1), CD86 (B7-2), and CD40 by resident and infiltrating host APCs. Under these circumstances, donor corneal APCs, which normally lack the capacity to stimulate T cells, become more potent in alloantigen presentation and priming of naïve T cells into Th1 effectors, the principal mediators of acute corneal graft rejection (see below for details of effector mechanisms) (10, 64, 65). Additionally, inflammation-induced expression of adhesion molecules and chemotactic gradient assist in mobilizing host APCs from the peri-corneal limbal vasculature centripetally toward the corneal graft (64, 66).

The cornea is normally devoid of both blood and lymphatic vessels. However, ocular inflammation leads to formation of blood and lymphatic neovessels. Inhibition of heme- and lymphangiogenesis in high-risk recipient mice has been shown to improve corneal allograft survival (43, 44, 67). The pathologic corneal lymphangiogenesis that occurs due to inflammation facilitates APC migration to draining lymph nodes, where they prime

alloreactive T cells (30). Expression of inflammatory cytokines, adhesion molecule ICAM-1, and CCL2 and CCL20 chemokines facilitate corneal infiltration of innate immune cells, which promote lymphangiogenesis through production of VEGF-C and VEGF-D (61, 68–71). It has been demonstrated that APCs, as a part of their “maturation” process also acquire VEGFR-3 expression, which in response to the chemotactic gradient of its principal receptor VEGF-C mediates APC trafficking to lymphoid tissues (72). APC migration through afferent lymphatic vessels is also dependent on the interaction between the CCR7 receptor and its ligand CCL21, which is expressed by lymphatic endothelial cells during inflammation, and APC interactions with ICAM-1 and VCAM-1 adhesion molecules (73, 74). The importance of lymphatic vessels in allosensitization is underscored by significantly higher APC trafficking and higher rejection rates seen in neovascularized or high-risk corneal graft beds that are lymphatic-rich compared to low-risk hosts (75, 76). Moreover, limiting the access of APCs to draining lymph nodes via ipsilateral cervical lymphadenectomy prior to transplantation has been shown to significantly prolong corneal allograft survival in murine models (77), providing proof of concept for the importance of lymphatics in facilitating allosensitization.

Allosensitization or priming of alloreactive T cells occurs via two distinct pathways. The *direct* pathway of allosensitization involves presentation of donor antigens in the context of non-self MHC by donor-derived APCs or “passenger leukocytes” to host naïve T cells (58). In the *indirect* pathway, however, host APCs recruited from the peripheral cornea (recipient bed) present donor antigens in association with self-MHC to naïve T cells in the draining lymph nodes (58). It was previously believed that the indirect pathway of allosensitization is the predominant, if not exclusive, form of immune response in all corneal transplants (78). However, identification of diverse populations of bone marrow-derived cells in the cornea that can acquire MHC class II expression under inflammatory conditions and thus serve as functional APC has further strengthened the functional role of direct alloreactivity in corneal transplantation (65, 79). Furthermore, accumulating data suggest that the relevance of direct or indirect pathways is highly dependent on graft bed microenvironment (58). In the non-inflamed setting where there is minimal expression of graft-derived MHC molecules, the indirect pathway remains predominant; however, in the high risk setting, characterized by graft bed inflammation and acquisition of high levels of MHC and accessory molecules by graft-borne APCs, the direct pathway is highly functional (Fig 2) (58, 80, 81). It has been demonstrated that the use of MHC class II-deficient donor tissue results in considerably prolonged survival of high-risk, but not low-risk corneal grafts (58), further underscoring the relevance of the direct pathway in high risk transplantation.

Effector immune cells and mechanisms of graft destruction

IFN γ -producing CD4⁺ Th1 cells are considered to be the predominant effector cells in corneal graft rejection (82, 83), but the precise mechanisms by which Th1 cells mediate graft rejection have not been fully elucidated. Although in vitro studies have demonstrated that alloreactive CD4⁺ T cells induce apoptosis in corneal endothelial cells (84), in vivo application of anti-FasL antibody or Fas-Fc protein does not inhibit CD4 T-cell mediated apoptosis of corneal cells (82). High expression of IFN γ and IL-2 in corneas undergoing rejection further supports the central role of Th1 cells in corneal allograft rejection (85, 86).

However, 33% of anti-CD4 antibody-treated mice and 45% of CD4 knock out mice still reject their corneal allografts, indicating that CD4-independent mechanisms are also involved in graft rejection (83, 85). In addition, studies on the rate of graft rejection in IFN γ knock out mice have demonstrated that 70% of MHC-mismatched grafts and none of MHC-matched grafts undergo rejection, suggesting that Th1 cells are not the sole mediators of graft rejection (87, 88).

It has long been proposed that skewing the immune system toward a Th2 alloimmune response promotes corneal allograft survival. Yamada et al. found that 'deviation' of the alloimmune response toward a Th2 phenotype promotes corneal allograft survival in a murine model of orthotopic corneal transplantation (89). However, recent studies have demonstrated higher rates of corneal allograft rejection in graft recipients with atopic (allergic) conjunctivitis, a disorder which is primarily mediated through a Th2 immune response (90, 91). Furthermore, it has been demonstrated that corneal graft rejection in IFN γ knock out mice is characterized by eosinophilic corneal infiltrates and is mediated by the Th2 pathway (87). Thus, in the aggregate, while there is general consensus that Th1 cells are the principal effectors of acute corneal graft rejection, it is clear that depletion of CD4 $^{+}$ T cells or IFN γ is unable to entirely suppress alloreactivity, thereby suggesting the involvement of myriad effector pathways including CD8 $^{+}$ and Th2 cells.

Involvement of Th17 cells has been established in the pathogenesis of multiple autoimmune diseases, including chronic ocular inflammatory conditions (92, 93). In transplant immunobiology, Th17 cells have been implicated in the development of lung and renal allograft rejection and graft-versus-host disease (94–96). Murine models of orthotopic corneal transplantation have revealed that the pathogenic role of Th17 cells is more evident at the very early stages of graft rejection, while IFN γ -producing Th1 cells are predominantly involved in later stages, and are critical for eventual graft rejection (97). The pro-lymphangiogenic role of Th17 cells has also been demonstrated in a murine model of autoimmune ocular surface inflammatory disease (98) indicating that blocking the effect of IL-17 may favor corneal allograft survival. However, approximately 90% of corneal allografts in IL-17 $^{-/-}$ mice or wild-type mice treated with anti-IL-17 antibody still reject their allografts (97, 99). Interestingly, IL-17 deficiency retards the development of alloimmune rejection in these hosts, and promotes the expression of Th2 type cytokines, IL-4, IL-5, and IL-13 (97). These data along with observations on corneal graft rejection in IFN γ knock out mice suggest that elimination of Th1 and Th17 pathways results in a Th2-biased immune response, and that deviating the host's immune response toward a Th2 phenotype, contrary to previous dogma, may in fact have a deleterious effect on corneal allograft survival (88, 97, 99). There has also been compelling evidence that IL-17 promotes the generation of CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ Tregs in corneal allografts, and is required for Tregs to exert their immunosuppressive function on effector CD4 $^{+}$ T cells (100).

Studies on the role of CD8 $^{+}$ or cytotoxic T cells in graft rejection have yielded controversial results as well. While some murine studies have suggested a role for donor-specific CD8 $^{+}$ T cells in high risk grafts, other studies have demonstrated that corneal allograft rejection occurs invariably in CD8 $^{+}$ T cell-deficient and perforin-deficient mice (101, 102). Overall results suggest that a CD8 $^{+}$ T cell response is not absolutely essential for corneal allograft

rejection. Although priming of CD8⁺ T cells occurs, in the absence of costimulatory signals from APCs, CD8⁺ T cells do not have the ability to reject the graft (103). CD8⁺ suppressor T cells (CD8⁺ Tregs), which are generated during the induction of ACAID, have been shown to suppress allospecific DTH response via perforin and FasL-independent mechanisms, promoting corneal immune privilege (104). Another subset of T cells, double negative or CD4⁻CD8⁻ T cells, has also been implicated in corneal graft rejection through apoptosis of corneal endothelium (102, 105). Adoptive transfer of CD4⁻CD8⁻ T cells to mice with severe combined immunodeficiency (SCID) results in acute corneal allograft rejection (102). However, the precise role of these cells as effector cells in mediating graft rejection is yet to be elucidated.

Allotolerance induction: contribution of regulatory T cells to allograft survival

One of the primary goals in transplant immunobiology is donor-specific tolerance induction which eliminates the need for immunosuppressive therapies and promotes graft survival. Tolerogenic Tregs and DCs are potential candidates that can be exploited for the induction of allotolerance in corneal transplantation. Maturation-resistant tolerogenic DCs (tolDCs), which express low levels of MHC class II and co-stimulatory molecules, have been implicated in suppression of alloimmunity and promotion of graft survival in multiple solid organ transplant models (106–108). Studies on murine models of penetrating keratoplasty have demonstrated that administration of donor-derived tolDCs to hosts prior to transplantation increases the frequencies of Foxp3^{hi} Tregs and significantly improves corneal allograft survival (109). Results of a recent study indicate that the beneficial effect of tolDCs on corneal graft survival is mediated through expansion of CTLA-4 expressing Tregs and downregulation of CD28⁺ Tregs (110). Given that tolDCs induce allotolerance primarily through Treg expansion, the majority of studies have focused on modification of Treg function to promote graft survival.

A myriad of studies have focused on the role of Tregs in allotolerance induction and in vitro expansion of Tregs to promote allograft survival (111, 112). Compelling evidence suggest that corneal allograft-induced donor specific Tregs are capable of suppressing the DTH immune response and enhancing allograft survival (99, 100, 112, 113). Generation and expansion of Tregs within the corneal allograft has been found to be mediated by the glucocorticoid-induced tumor necrosis factor receptor family-related protein ligand (GITRL), which is constitutively expressed in the cornea (114). Studies on the suppressive function of Tregs have revealed that Treg-mediated suppression of effector T cells is primarily contact-dependent and mediated by membrane-bound GITRL and CTLA-4 molecules (100). Additionally, IL-17 regulates the expression of Foxp3 and these membrane-bound molecules on Tregs (100). As mentioned above, IL-17 is required for the generation of Tregs, and treatment with an anti-IL-17 antibody results in rejection of 90% of corneal allografts (100). Although previously non-ocular allograft survival was associated with increased frequencies of Tregs within the graft or the draining lymph nodes (115), a recent study has reported no difference in frequencies, but higher levels of Foxp3 expression in the draining lymph node Tregs of accepted corneal allografts compared to allografts undergoing rejection (Fig 3A) (116). The suppressive function of Tregs has been related to their expression of Foxp3 (Fig 3B) (116, 117). Moreover, it has been demonstrated that

Foxp3^{hi} Tregs from accepted grafts are more potent in suppressing naïve T cell proliferation and secreting IL-10 and TGF- β (116). Studies on homing of Tregs to draining LNs of corneal allograft recipients and their interaction with APCs (Fig 3C) have shown that: (i) Tregs from allograft acceptors localize in the paracortical region of draining LNs in close proximity with APCs and express higher levels of CCR7, while Tregs from graft rejectors express lower levels of CCR7 and are less in contact with APCs, (ii) CCR7^{hi} Tregs have a more significant inhibitory effect on T cell proliferation and secrete higher levels of immunosuppressive cytokines, and (iii) in vitro stimulation of naïve Tregs with CCL21 upregulates their CCR7 expression and improves Tregs' homing ability to draining lymph nodes and significantly enhances corneal allograft survival (Fig 3D) (118). These data accumulatively suggest that Tregs become dysfunctional in grafts undergoing rejection. A recent study from our laboratory has shown that Treg dysfunction can be prevented by systemic administration of low-dose IL-2. Our data have demonstrated that systemic treatment of high risk recipient mice with low-dose IL-2 results in expansion and improved suppressive function of Tregs, reduced leukocyte infiltration of graft, and significantly improved corneal allograft survival (119).

Instability of Tregs or "Treg plasticity" in the inflammatory environment is a new concept that has recently gained attention. Recent data suggest that in the inflamed setting a significant number of Tregs exhibit unstable expressions of Foxp3; these "exTregs" acquire an effector memory T cell phenotype and produce IFN γ , which may further contribute to the development of autoimmunity (120). Recently our laboratory has employed a model of corneal transplantation in Foxp3-lineage reporter transgenic mice to evaluate the pathologic conversion of Tregs in immune privilege-disrupted (high risk) hosts. The results have suggested that ocular inflammation results in conversion of Tregs into IFN γ -producing exTregs. Neuropilin-1 (Nrp-1), a membrane-bound coreceptor which is selectively expressed on natural/thymic Tregs (tTregs) and not on peripherally induced Tregs (pTregs), was used to further determine the lineage of these exTregs (121). Our results demonstrated that Nrp-1⁻ pTregs are more susceptible than Nrp-1⁺ tTregs to the effects of pro-inflammatory cytokines expressed in inflamed host beds, mediating their conversion into exTregs (unpublished data). These data suggest that the pathologic conversion of Tregs and their impaired function contribute to loss of corneal immune privilege and allograft rejection.

Conclusions

Corneal allograft rejection is a highly complex process that involves an elaborate interplay between cells of the innate and adaptive immunity and the lymphovascular system. Our understanding of immunology and pathophysiology of corneal allograft rejection has been considerably improved in recent years, and many of the recent investigations have focused on development of new therapies that could target the afferent and efferent arms of immunity at a molecular level, without compromising the integrity of the immune system. However, the redundancy of cellular and molecular pathways mediating graft rejection has made this a daunting task. Evolving strategies for allotolerance induction, primarily regulatory T cell-based therapies are promising tools that could bring us closer to safe therapeutic modalities for corneal graft rejection.

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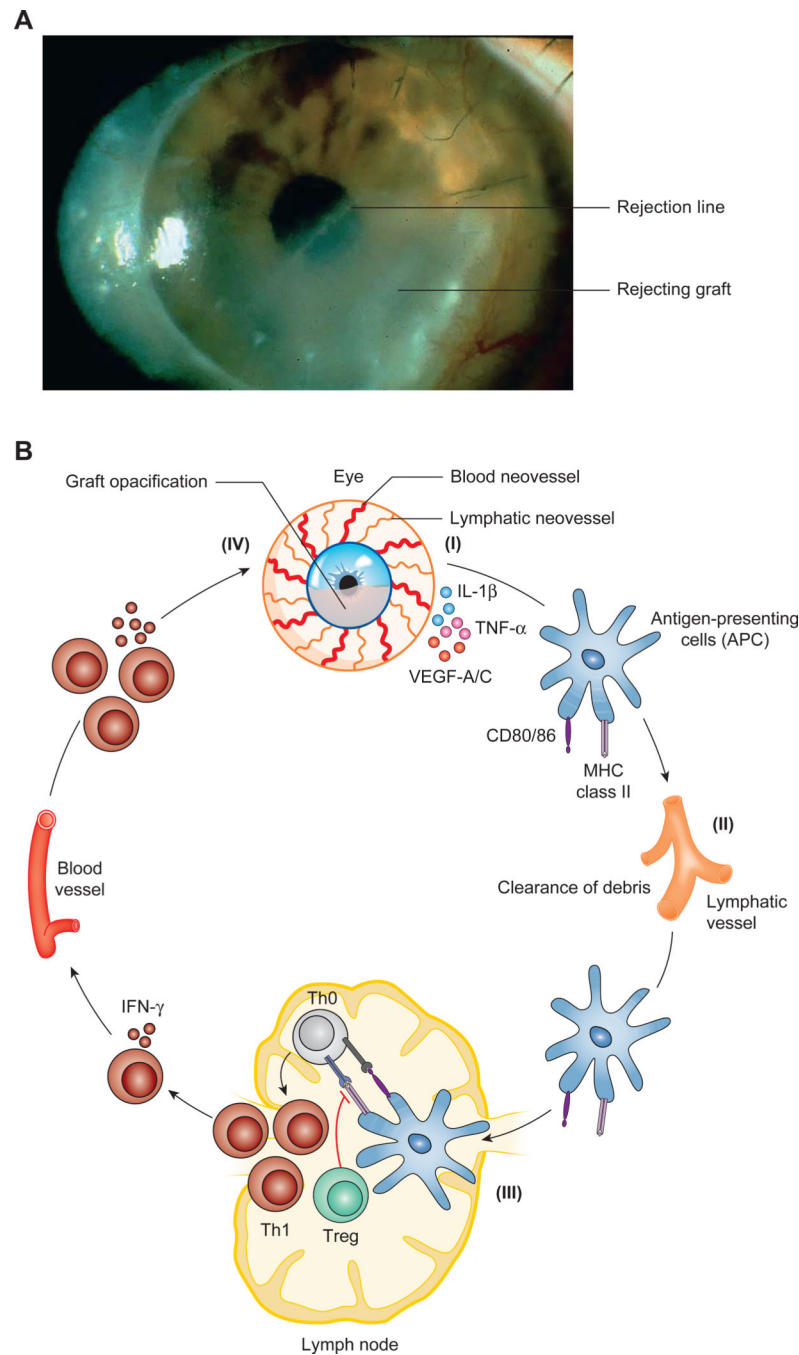


Figure 1. Immunobiology of corneal transplantation

A. Clinical manifestation of corneal graft rejection. Infiltrating monocytes and T cells attack the graft, often involving a rejection line that marches across the inner endothelial layer of the transplant, leaving an opaque and swollen graft behind. **B.** Schematic representation of corneal alloimmunity **(I)**. Following transplant surgery, upregulation of pro-inflammatory cytokines, adhesion molecules and pro-angiogenic factors results in corneal infiltration of immune cells and formation of new blood and lymphatic vessels. **(II)**. Antigen presenting cells (APCs), which acquire MHC class II and co-stimulatory molecules in the inflammatory

environment, egress from the cornea through lymphatic vessels to the draining lymph nodes, where they present alloantigens to naïve T cells (Th0). Newly formed lymphatic vessels may also contribute to resolution of inflammation by mediating clearance of inflammatory cells and debris. **(III)**. Primed T cells undergo clonal expansion and differentiate primarily into IFN γ -secreting CD4⁺ Th1 cells. Regulatory T cells (Tregs) modulate induction of alloimmune response either through inhibition of T cell activation or suppression of APC stimulatory potential. **(IV)**. Alloreactive Th1 cells, migrate through blood vessels and along a chemokine gradient toward the graft, where they mount a delayed-type hypersensitivity response against the allogeneic tissue, resulting in graft opacification and failure.

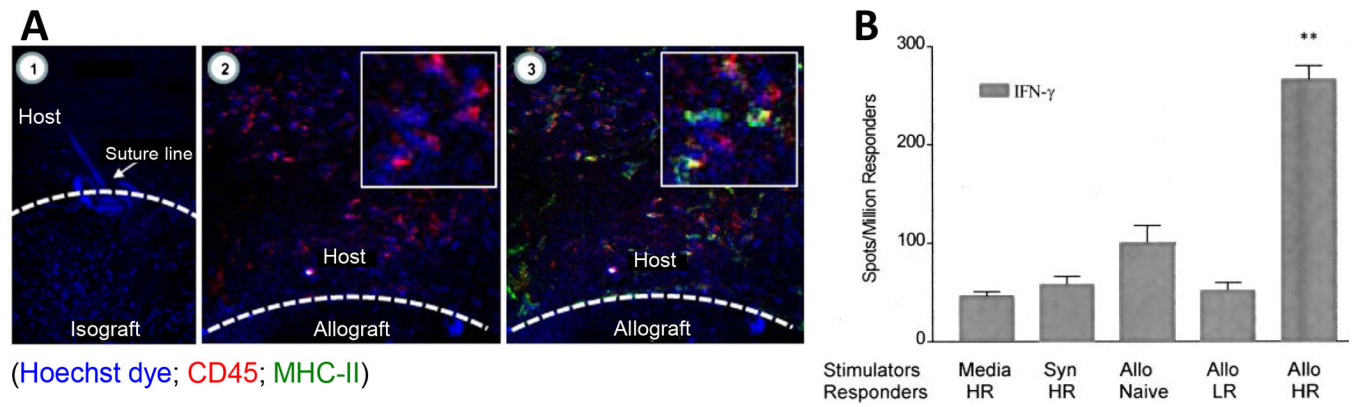


Figure 2. Migration of donor graft-derived APCs and activation of the direct pathway of allosensitization

A. Ex vivo staining of corneal grafts with Hoechst vital dye tracks the egress of donor APCs posttransplantation. Stained isografts (BALB/c \rightarrow BALB/c) demonstrates that ex vivo staining (blue; A1) is mostly restricted to the graft and not the host bed. In contrast, stained allografts (C57BL/6, IAb \rightarrow BALB/c, IAd) evaluated at 24 h posttransplantation demonstrate that exiting donor cells in the host bed are largely CD45⁺ (red; A2) and express donor IAb (green; A3) (inlays are respective digitally enlarged portions of host beds). **B.** The frequencies of IFN γ -producing T cells 2 weeks after transplantation were assessed using ELISPOT. In high risk (HR) graft recipients, a significant higher IFN γ response is generated in directly primed allospecific T cells compared to low risk (LR) recipients and ungrafted controls (Naïve) (** $p < 0.01$), suggesting that the direct alloresponse is the predominant form of allosensitization in the high-risk graft setting. *Adopted and modified from Saban, D. R., et al.²⁵ & Huq, S., et al.⁵⁸*

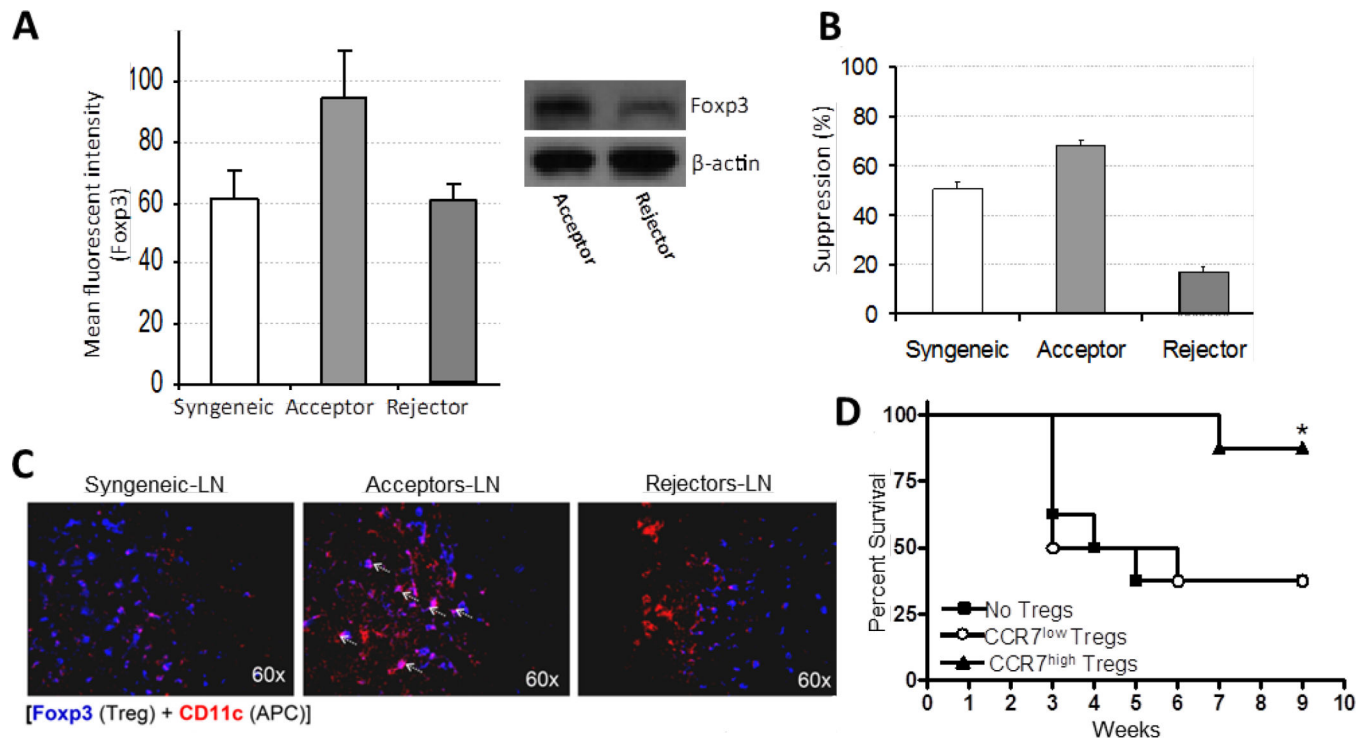


Figure 3. Treg function and their interactions with antigen presenting cells in the draining lymph nodes of corneal allograft acceptors versus allograft rejectors

A. Mean fluorescent intensity (MFI) and Western blot analysis of Foxp3 expression in CD4⁺CD25⁺Foxp3⁺ Tregs from draining lymph nodes of allograft acceptors and rejectors 3 weeks posttransplantation demonstrates significantly higher expression levels of Foxp3 in Tregs from graft acceptors compared to Tregs from grafts undergoing rejection. **B.** Tregs isolated from the lymph nodes of graft acceptors are significantly more potent in suppressing the proliferation of CD3-stimulated naïve T cells compared to Tregs isolated from lymph nodes of graft rejectors and syngeneic recipients. **C.** Confocal micrographs of draining lymph nodes show that only Tregs from graft acceptors colocalize with CD11c⁺ APCs (white arrows, 60×). **D.** Adoptive transfer of CCR7^{hi} Tregs into corneal allograft recipients significantly improves corneal allograft survival (* $p=0.022$). Allograft recipients that receive CCR7^{lo} Tregs demonstrate no improvement in allograft survival. *Adopted and modified from Chauhan, S. K., et al.*^{116, 118}

Table 1

Factors involved in immune and angiogenic privilege of the cornea

Factor*	Function	Reference
Angiostatin	Inhibits vascular endothelial cell (VEC) proliferation	45
α -MSH	Suppresses IFN γ production by T cells, Promotes Treg development	33, 38
CGRP	Suppresses NO production by macrophages	35
CRP	Inhibits activation of the complement system	32
Endostatin	Promotes VEC apoptosis	46
FasLigand	Promotes apoptosis of PMNs and T cells	26
IDO	Promotes T cell apoptosis, Suppresses NK cell proliferation	39
IL-1Ra	Suppresses APC migration	23
MIF	Inhibits NK cell-mediated cytotoxicity of MHC class I-negative cells	36, 37
PDL-1	Promotes T cell apoptosis, inhibits T cell proliferation and IFN γ production	21, 22
PEDF	Suppresses VEGF expression	47
TGF- β	Inhibits NK cell-mediated cytotoxicity of MHC class I-negative cells, Suppresses T cell activation	31
TRAIL	Promotes T cell apoptosis and proliferation of Tregs	27–29
TSP-1	Inhibits APC maturation and migration, and T cell allosensitization	25
VEGFR-1	Inhibits the mitogenic activity of VEGF-A on VECs	40
VEGFR-2	Inhibits the angiogenic activity of VEGF-C	44
VEGFR-3	Inhibits hemangiogenesis and lymphangiogenesis, decoy non-signaling receptor for VEGF-C and VEGF-D	41, 43
VIP	Suppresses T cell activation and proliferation	34

* Listed alphabetically; α -MSH, alpha-melanocyte stimulating hormone; CGRP, calcitonin gene-related peptide; CRP, complement regulatory proteins; IDO, indoleamine 2,3-dioxygenase; IL-1Ra, interleukin-1 receptor antagonist; MIF, macrophage migration inhibitory factor; PDL-1, programmed death ligand-1; PEDF, pigment epithelium-derived factor; TGF- β , transforming growth factor-beta; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TSP-1, Thrombospondin-1; VEGFR, vascular endothelial growth factor receptor; VIP, vasoactive intestinal peptide.